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Renoprotective effect of low iron diet and its consequence on glomerular hemodynamics

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Renoprotective effect of low iron diet and its consequence on glomerular hemodynamics. It has been reported that anemia limits renal injury in rats with reduced renal mass. We studied the effect of a low iron diet, given to reduce hematocrit, on urinary protein excretion and glomerular function in male MWF/Ztm rats, which spontaneously develop proteinuria and glomerular sclerosis. At 20 weeks of age, micropuncture and glomerular volume measurements were performed in untreated rats fed standard chow and in rats fed an isocaloric diet with low iron (5 mg/kg) content. Two additional groups of rats were used for total kidney function and glomerular volume evaluation at 35 weeks of age. At 20 weeks of age animals on low iron diet showed significantly ($P < 0.01$) reduced hematocrit (46 ± 5% vs. 54 ± 2%) and proteinuria (60 ± 15 vs. 225 ± 34 mg/24 hr) than control animals, and no statistically significant differences were observed in single nephron hemodynamics. At 35 weeks of age rats on low iron diet had significantly lower proteinuria than age matched controls (222 ± 68 vs. 411 ± 71 mg/24 hr, $P < 0.01$) and developed less glomerular sclerosis (mean percentage of sclerotic glomeruli was respectively 14 ± 7% and 31 ± 17%, $P < 0.05$). Glomerular volume was comparable in animals on the low iron diet and in controls both at 20 and 35 weeks of age. These data indicate that low iron diet protected male MWF/Ztm rats against glomerular injury without significant effects on glomerular hemodynamics and on glomerular volume.

Several studies have been performed in the last few years with the aim to understand the progressive nature of renal disease which follows immunologic or toxic glomerular injury. One of the most studied models of renal disease progression is partial ablation of renal mass in the rat that leads to systemic hypertension, abnormal glomerular permeability to proteins and glomerulosclerosis [1]. The reduced number of functioning nephrons in the above-mentioned model determines an increase in glomerular filtration rate (GFR) and in capillary hydraulic pressure (P_{GC}) in remnant glomeruli [2]. That glomerular hypertension plays a crucial role in the progression of renal lesions when the number of nephrons is reduced has been documented by two factors. First, dietary protein restriction, which reduces P_{GC}, provides a sustained protection against the progression of glomerular lesions [2, 3]; secondly a peculiar class of antihypertensive agents, angiotensin I converting enzyme inhibitors (CEI), limits glomerular capillary hypertension and prevents glomerular sclerosis. Antihypertensive agents that do not modify glomerular capillary hypertension have no effect on progressive glomerular damage [4]. Whether in models in which the number of functioning nephrons is not reduced either by surgical removal of renal mass or by a pre-existing disease (as in streptozotocin diabetes), factors other than glomerular capillary hypertension which may contribute in altering glomerular permeability to proteins and glomerular sclerosis are, however, less clear.

Recently Garcia and coworkers [5] have documented the potential role of hematocrit (Hct) in modulating glomerular hemodynamics and renal disease progression. Specifically they have found that in animals with reduced renal mass anemia reduces glomerular injury while raising Hct values by administration of recombinant human erythropoietin accelerates glomerular sclerosis. They have suggested that anemia retards the development of proteinuria and glomerular injury by control of systemic and glomerular hypertension.

We have previously studied the evolution of glomerular injury in a strain of rats, originally selected from Munich Wistar rats by Fromter (MWF/Ztm) for its high number of superficial glomeruli [6, 7]. Males of these strain develop spontaneous proteinuria and glomerular sclerosis. In this model glomerular damage develops spontaneously; thus it represents an alternative condition for studying renal disease progression perhaps more close to some human renal disease.

The present study was designed with two aims: a) to establish whether reduction in Hct protects against the development of glomerular injury when the number of functioning nephrons is not reduced; b) to elucidate the possible pathophysiological basis of the protective effect of anemia in such circumstances.

Methods

Reduction of dietary iron content and periodic phlebotomy were used to reduce Hct in MWF/Ztm rats as shown previously [5]. Rats, originally supplied by H. Hackbarth from Hannover, were bred and raised in our facilities [6, 8]. Two groups of animals, group 1 ($N = 8$) and group 3 ($N = 7$), received standard rat chow (120 mg of iron per kg of chow; Rieper, Bolzano, Italy) throughout the study and are referred to as control groups. Two other groups of rats (group 2, $N = 8$, and group 4, $N = 7$) received a low iron diet (5 mg of iron per kg of chow) from the fifth week of age throughout the study, and were subjected to

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periodic phlebotomy. The two diets were isocaloric and had the same protein content (19%). Hematocrit and systolic blood pressure were measured periodically as previously described [5, 9]. Urinary protein excretion, food and water intake in 24 hours were determined periodically in all animals using metabolic cages. Protein concentration in urine was determined by the Coomassie blue G-dye-binding method [10].

Rats of groups 1 and 2 underwent micropuncture study at 20 weeks of age. Animals of groups 3 and 4 were followed for additional 15 weeks, at which time (35 weeks) whole-kidney function was measured by clearance technique. In all animals kidney tissue was processed for morphological evaluation of renal damage and for glomerular volume (V_G) determination.

Micropuncture studies

Single glomerular hemodynamics were studied using micropuncture technique as previously described [7]. Briefly, anesthetized rats (Inactin, 100 mg/kg body wt), were placed on a constant temperature micropuncture table and tracheostomized. The left femoral artery was catheterized for blood sampling and for continuous arterial pressure recording (Battaglia, Rangoni, Bologna, Italy). Polyethylene catheters were inserted into the left jugular and left femoral vein for infusion of inulin solution (7%, dissolved in 0.9% NaCl) and isoncotic rat plasma. The left kidney was then exposed through a subcostal incision, a polyethylene catheter was inserted into the left ureter, and the kidney was placed in a Lucite holder and bathed with isotonic NaCl. Isoncotic rat plasma was infused during the experiment to compensate surgically induced loss of plasma volume as previously described [11].

After one-hour equilibration period, exactly timed proximal tubular fluid samples were collected from five to six separate nephrons using sharpened glass pipettes. Three to five samples of efferent arteriolar blood were withdrawn from superficial star vessels using sharpened oil-filled glass pipettes. Time-averaged hydraulic pressure was measured in surface glomerular capillaries, in proximal tubules, and in first order peritubular capillaries using sharpened glass pipettes and a servo-nulling micropipet pressure system (Model 5, I.P.M., San Diego, California, USA). During micropuncture measurements three timed urine collections of 20 to 30 minutes each were performed. Blood samples were obtained at the midpoint of each clearance period for determination of Hct and plasma concentrations of inulin and total proteins.

The volume of tubular fluid samples was measured from the length of the fluid column in a constant bore capillary tube of known diameter. Inulin concentration in tubular fluid was determined by the method of Vurek and Pegram [12]. Protein concentrations in efferent arteriolar and femoral arterial plasma samples were determined, using an ortho-phthalaldehyde technique adapted for small sample volumes [13]. Inulin and total protein concentrations in plasma and urine samples were measured using methods previously described [14, 15]. GFR was calculated as inulin clearance using standard formula. Calculations of single nephron (SN) GFR, colloid osmotic pressure in afferent and efferent arterioles (π_a, π_e), glomerular afferent plasma flow (Q_a), afferent and efferent arteriolar resistance (R_a, R_e) were done using equations described previously in detail [16]. Glomerular ultrafiltration coefficient (K_f), the product of glomerular hydraulic membrane permeability and filtering surface area, was calculated according to the model of glomerular ultrafiltration of Deen, Robertson and Brenner [17].

Whole-kidney functional studies

Total kidney GFR and renal plasma flow (RPF) were determined using inulin and para-aminohippuric acid (PAH) clearance as previously described [7]. Briefly, rats were anesthetized as described for micropuncture study, placed on a constant-temperature table and polyethylene tubing was inserted in the jugular vein for inulin and PAH infusion. The right femoral artery was cannulated for continuous recording of arterial pressure. Urine was collected by bladder cannulation. After a 40 to 50 minute equilibration period three timed urine collections were started; blood samples were obtained from the femoral artery at the midpoint of each clearance period. PAH concentrations in plasma and urine samples were determined with the method of Smith et al [18].

Morphological studies

After micropuncture or whole-kidney function studies the left kidney was fixed by perfusion, at the measured arterial pressure, with 1.25% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4). After fixation two midcoronal sections of the kidney were postfixed in Dubosq-Brazil fluid and embedded in paraffin [19] for light microscopy. Sections 3 μm in thickness were stained with Masson's trichrome, hematoxylin and eosin, and by the periodic-acid Schiff techniques. Sections including superficial and juxtamedullary glomeruli were evaluated. At least 100 glomeruli were examined for each animal and the percentage of glomeruli affected by focal or global glomerular sclerosis was determined.

V_G determination was performed as previously described [7]. Images of histological sections were projected using a light microscope and a drawing tube. The outline of at least 50 systematically sampled glomeruli per animal were manually traced. The area of the capillary tuft outlines were measured using a computer based image analysis system [7]. Average V_G of individual animals was calculated from mean glomerular tuft cross-sectional area as described [7, 20].

Statistical analysis

All results are expressed as mean ± standard deviation (SD). Data were analyzed using Student's *t*-test for unpaired data and two-way analysis of variance as appropriate. Significant differences between individual group means, subjected to the analysis of variance, was established using Tukey-Cicchetti test for multiple comparisons [21]. Estimates of glomerular sclerosis incidence were compared with Mann-Whitney test for non-parametric data. Statistical significance was defined as $P < 0.05$.

Results

As shown in Figure 1 male MWF/Ztm rats on the low iron diet showed significantly reduced Hct as compared to rats receiving the standard diet. Anemia was more pronounced at 10 weeks of age, when Hct values progressively increased. By the end of the observation period (35 weeks) animals on low iron diet had Hct values still numerically lower than control animals but the difference was not statistically significant. Control animals developed systemic hypertension; mean systolic blood

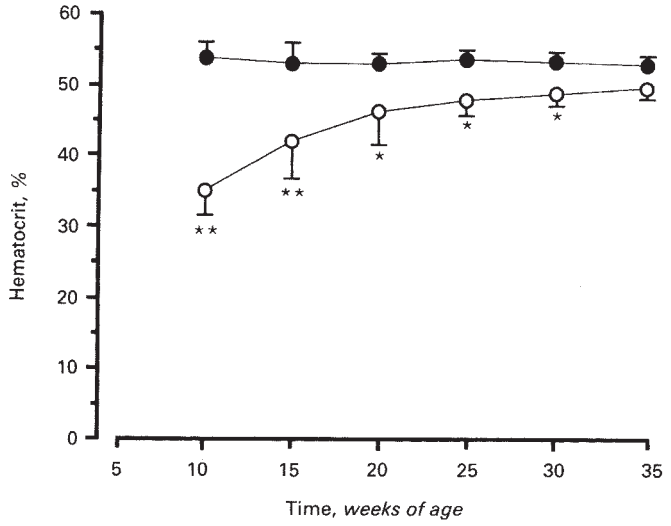

Fig. 1. *Hematocrit values in conscious rats on standard (group 3) and low iron diet (group 4) from 10 to 35 weeks of age.* Values are means ± SD. * $P < 0.05$ vs. standard diet, ** $P < 0.01$ vs. standard diet.

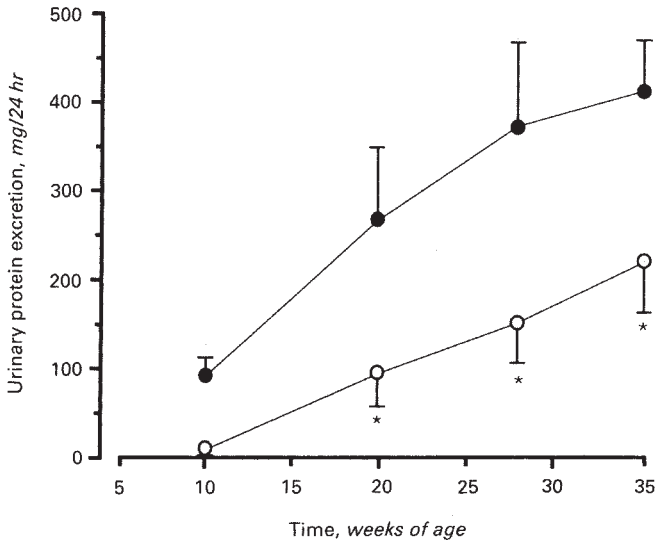

Fig. 2. *Urinary protein excretion rate measured in rats on standard (group 3) and low iron diet (group 4) from 10 to 35 weeks of age.* Values are means ± SD. * $P < 0.01$ vs. standard diet.

pressure was 153 ± 14 mm Hg at 10 weeks, and reached the mean value of 186 ± 17 mm Hg by the end of the study (35 weeks). At 10 weeks of age, mean systolic blood pressure in animals on low iron diet was comparable to the control group (153 ± 9 vs. 153 ± 14 mm Hg, respectively, groups 3 vs. 4). By the end of the observation period systolic blood pressure in animals receiving the low iron diet was numerically reduced, as compared to controls, but the difference was not statistically significant (167 ± 14 vs. 186 ± 17 mm Hg, groups 3 vs. 4).

Urinary protein excretion rates measured during the observation period are reported in Figure 2. Untreated rats developed massive proteinuria, reaching the value of 411 ± 71 mg/24 hr by 35 weeks of age. Low iron feeding significantly reduced urinary protein excretion rate as compared to rats on standard diet at the same time point. Proteinuria was completely prevented in animals on low iron diet at 10 weeks of age (Fig. 2), then urinary protein excretion increased, reaching the mean value of 222 ± 68 mg/24 hr by the end of the study (35 weeks). Values of Hct, systolic blood pressure and urinary protein excretion in animals of groups 1 and 2, which underwent micropuncture studies at 20 weeks of age (Table 1), were respectively comparable to those observed in animals of group 3 and 4 (Figs. 1 and 2) at the same age.

Micropuncture studies

Mean values of whole-kidney and single-nephron functional parameters measured in groups 1 and 2 are summarized in Table 1. Despite comparable food and water intake, measured periodically during the observation period, body and kidney weight in animals fed the low iron diet were lower than in controls, however, the differences were not statistically significant. As previously reported for animals of groups 3 and 4 (Figs. 1 and 2), urinary protein excretion and Hct were significantly reduced in animals on the restricted iron diet as compared to control rats. Mean AP under anesthesia was significantly reduced on low iron diet (group 2) as compared to standard diet (group 1). Total kidney GFR was numerically higher in control animals than in rats receiving the low iron diet, although the difference did not reach statistical significance. Mean hydraulic pressure in glomerular capillaries (P_{GC}) and in proximal tubules (Pt) were comparable in both animal groups, thus no significant differences were calculated for transmural pressure difference (ΔP). Also values for hydraulic pressure in first order peritubular capillaries as well as afferent and efferent arteriolar plasma protein concentration and colloid osmotic pressure were comparable in the two groups (Table 1).

As observed for total kidney GFR, mean SNGFR was numerically lower in animals on low iron diet than in controls but the difference was not statistically significant. Mean Q_a was comparable in the two groups, while SN filtration fraction (FF) was significantly reduced in animals on low iron diet as compared to animals on standard diet ($P < 0.05$, Table 1). Afferent and efferent arteriolar resistances tended to be lower in animals fed the low iron diet, as compared to control animals, but the difference was not statistically significant. Mean calculated values of ultrafiltration coefficient (K_f) were comparable in both animal groups.

Morphological studies

At 20 weeks of age animals on standard and on low iron diets (groups 1 and 2) tended to develop focal and segmental glomerular sclerosis. Mean number of glomeruli affected by sclerotic changes was comparable in both groups (5 ± 4% and 4 ± 3% in controls and in animals on low iron diet, respectively) and the extent of sclerotic lesions was mild, involving less than 10% of glomerular tuft area. However, by the end of the study (35 weeks) the incidence of glomerulosclerosis in animals on standard diet was significantly higher than in rats receiving the low iron diet; the mean percentage of sclerotic glomeruli was 31 ± 17% and 14 ± 7%, group 3 versus group 4, respectively ($P < 0.05$). None of the glomeruli examined showed global sclerosis in both groups 3 and 4, however, the severity of segmental

Table 1. Whole-kidney and single nephron functional parameters in animals on standard and low iron diet at 20 weeks of age

Group	Body wt	Kidney wt	$U_{prot}V$	Hct	$\overline{AP}$	GFR	P_{GC}	P_t	ΔP	P_e
	g		*mg/24 hr*	*%*	*mm Hg*					
1 Standard diet ($N = 8$)	378 ± 24	1.25 ± 0.08	225 ± 34	51 ± 2	138 ± 9	0.71 ± 0.17	51 ± 2	12 ± 1	39 ± 2	21 ± 1
2 Low iron diet ($N = 8$)	358 ± 24	1.12 ± 0.19	60 ± 15[b]	45 ± 3[b]	119 ± 8[b]	0.62 ± 0.15	50 ± 2	13 ± 1	37 ± 2	21 ± 1

Values are mean ± SD.

Abbreviations are: $U_{prot}V$, urinary protein excretion rate; Hct, hematocrit; $\overline{AP}$, mean arterial pressure during micropuncture; P_{GC}, glomerular capillary hydraulic pressure; P_t, proximal tubule hydraulic pressure; P_e, efferent arteriolar hydraulic pressure; C_a, C_e, afferent and efferent arteriolar plasma protein concentration; π_a, π_e, afferent and efferent colloid osmotic pressure; Q_a, single glomerular afferent plasma flow; R_a, R_e, afferent and efferent arteriolar resistance; R_t, total arteriolar resistance ($R_a + R_e$).

[a] $P < 0.05$ vs. group 1

[b] $P < 0.01$ vs. group 1

Table 2. Whole-kidney functional parameters in animals on standard and on low iron diet at 35 weeks of age

Group	Body wt	Kidney wt	$U_{prot}V$	Hct	$\overline{AP}$	C_a	GFR	RPF	FF
	g		*mg/24 hr*	*%*	*mm Hg*	*g/dl*	*ml/min*		
3 Standard diet ($N = 7$)	408 ± 41	1.49 ± 0.21	411 ± 71	51 ± 2	146 ± 11	6.2 ± 0.4	1.29 ± 0.16	6.7 ± 1.2	0.20 ± 0.05
4 Low iron diet ($N = 7$)	412 ± 24	1.43 ± 0.11	222 ± 68[a]	49 ± 2	146 ± 15	5.8 ± 0.3	1.22 ± 0.12	7.5 ± 2.2	0.16 ± 0.05

Values are mean ± SD.

Abbreviations are: $U_{prot}V$, urinary protein excretion rate; Hct, hematocrit; $\overline{AP}$, mean arterial pressure; C_a, arterial plasma protein concentration; GFR, glomerular filtration rate; RPF, renal perfusion factor; FF, filtration factor.

[a] $P < 0.01$ vs. group 3

sclerotic lesions was different in the two groups. As shown in Figure 3, whereas in control animals (group 3) sclerotic lesions involved a substantial part of glomerular tuft area (from 20 to 50%), in animals on low iron diet (group 4) the sclerotic lesions never involved more than 20% of the glomerular tuft. No differences were noted in the distribution and extent of sclerotic changes between cortical and justaglomerular glomeruli.

Morphometrical analysis of glomerular capillary tufts showed comparable values of mean V_G between animals on standard and on low iron diet both at 20 weeks of age (0.87 ± 0.18 and 0.80 ± 0.19 $\mu m^3 \times 10^{-6}$ groups 1 and 2, respectively) and at 35 weeks (1.09 ± 0.11 and 1.16 ± 0.21 $\mu m^3 \times 10^{-6}$, groups 3 and 4).

Discussion

Our results demonstrated that in MWF/Ztm rats dietary iron deficiency, aimed to reduce Hct, significantly protected against the abnormal glomerular permeability to proteins and glomerular sclerosis that develop with age in normal males of this strain. In animals maintained on low iron diet, urinary protein excretion significantly increased with age, but remained significantly lower than in control animals until the end of the observation period.

Despite the low iron diet and periodic phlebotomy, important anemia was observed only at 10 weeks of age, then Hct progressively increased and was comparable to control values at the end of the study. Previous experiments have shown [5] that in rats with renal mass reduction, the Hct level had profound effects on systolic blood pressure and glomerular hemodynamics, and specifically that a relatively minor reduction in Hct had a major effect on P_{GC}. It was proposed that the effect of anemia of limiting systemic and glomerular hypertension was an explanation for the favorable effect of proteinuria and glomerular injury. In the present study control animals showed systemic hypertension. In animals on low iron diet, systolic blood pressure was numerically lower than in controls, but still higher than normal. This indicates that other factors beside blood pressure reduction must be involved in the protective effect of low iron diet. Male MWF/Ztm animals, unlike animals with renal mass ablation, have normal glomerular capillary pressure. It seemed important, therefore, to investigate whether the protective effect of lowering Hct in the latter model was associated with glomerular hemodynamic changes. Findings of comparable SNGFR, and all its determinants (Q_a, ΔP, K_f, C_a) in experimental and control animals would apparently exclude a hemodynamic explanation for the observed protective effect of the low iron diet in MWF/Ztm rats.

Since micropuncture studies were performed in animals of 20 weeks of age one could wonder whether glomerular hemodynamic changes may have developed later in time (that is, at 35 weeks of age). However, it is unlikely that low iron feeding influences glomerular hemodynamics at 35 weeks but not at 20 weeks, when Hct was significantly reduced compared to control animals and protection against proteinuria was maximal.

Recent studies have advocated glomerular volume expansion as one of the causative factors for the progression of glomerular disease [22]. In particular, it has been suggested that glomerular hypertrophy is an important step preceding experimental glomerular sclerosis [23]. Maneuvers that prevent glomerular injury, such as the use of ACE inhibitors, also prevented glomerular volume expansion [24]. We then investigated whether the protective effect of the low iron diet observed in

Table 1. *Continued*

C_a	C_e	π_a	π_e	SNGFR	SNFF	Q_a	$R_a \times 10^{-10}$	$R_e \times 10^{-10}$	$R_t \times 10^{-10}$	K_f
g/dl		*mm Hg*		*nl/min*			*dyne/sec/cm^{-5}*			*nl/sec/mm Hg*
5.4 ± 0.3	7.8 ± 0.9	17 ± 2	30 ± 5	44.3 ± 5.4	0.31 ± 0.06	152 ± 45	2.37 ± 0.61	0.98 ± 0.26	3.36 ± 0.85	0.049 ± 0.009
5.4 ± 0.4	7.1 ± 0.4	17 ± 2	27 ± 2	38.8 ± 4.6	0.24 ± 0.05[a]	168 ± 45	1.89 ± 0.53	0.92 ± 0.26	2.86 ± 0.86	0.044 ± 0.009

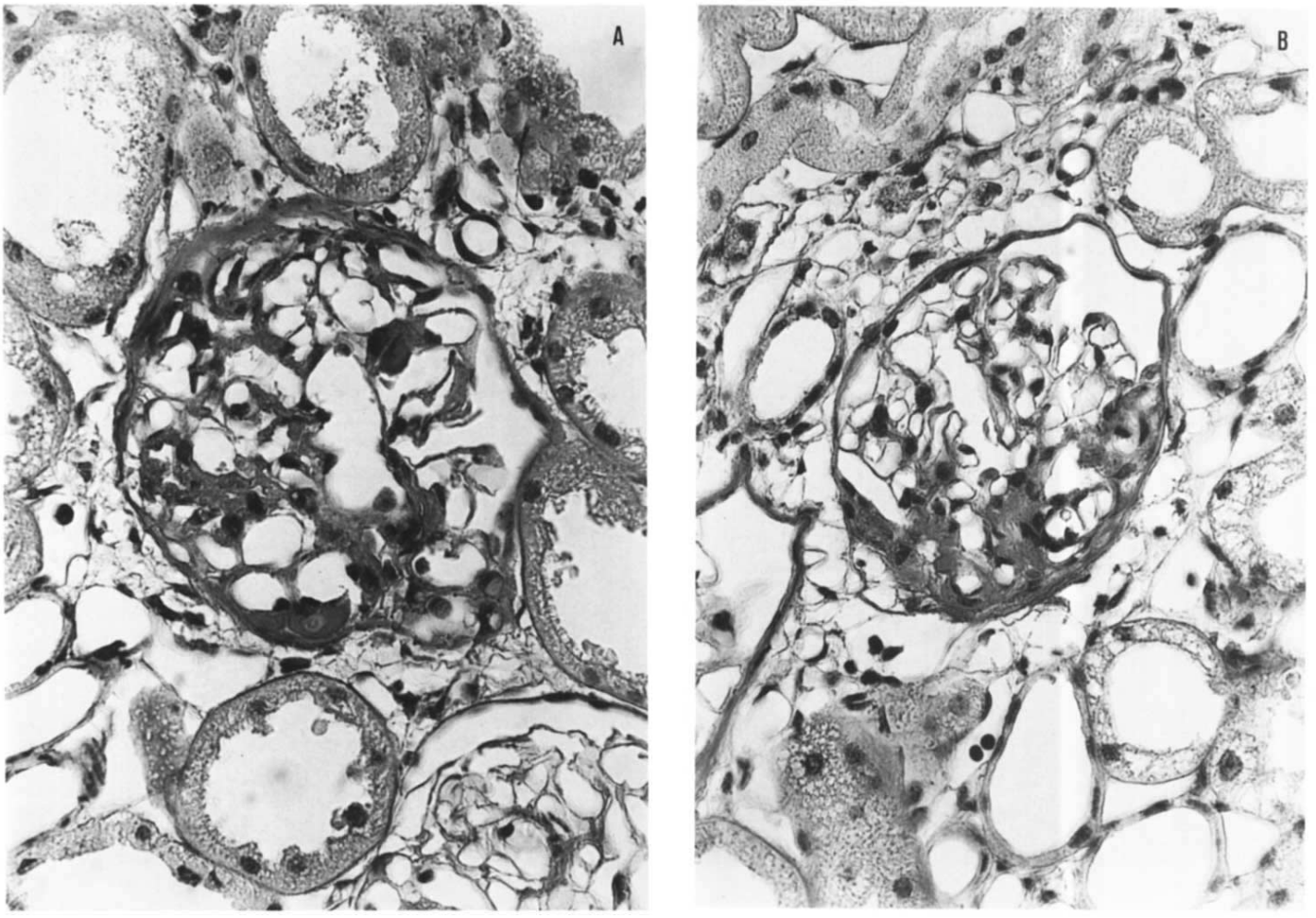

Fig. 3. *Light microscopy of representative glomeruli from a control animal* (**A**, *group 3) and for an animal on low iron diet* (**B**, *group 4) at 35 weeks of age*. More extensive glomerular sclerotic lesions are present in the control rat (A) than in the rat maintained on low iron diet (B).

our study was associated with changes in V_G. However, experimental and control rats had comparable values of mean V_G both at 20 and 35 weeks of age. These findings are in keeping with previous data, in that two months of treatment with a CEI protected male MWF/Ztm rats from proteinuria and glomerulosclerosis with no effect on glomerular volume [7]. Altogether these data might be taken to indicate that the observed protective effect of anemia on glomerular injury is not directly related to factors that modulate glomerular expansion.

Alternative possibilities to explain our findings include: (i) changes in hemorheology of glomerular microcirculation and (ii) effects of the iron deficient diet on the local generation of oxygen free radicals. Concerning the first possibility one has to realize that Hct is a major determinant of blood viscosity and ultimately of hemodynamic shear stress upon glomerular capillary wall [25]. It has been reported that changes in fluid shear stress on vascular endothelium influence endothelial cell functional properties to such an extent that factors are generated locally that may alter vascular tone [26] as well as the interaction between circulating cells and glomerular capillary wall [27]. These factors could play a role in mediating glomerular capillary wall damage. On the same line an increase in blood viscosity has been reported to enhance the activity of the renin-angiotensin system [28], which might then be suppressed by lowering blood viscosity. Of interest in this context, both the use of a CEI [7] and reduction of Hct afford protective effects against renal injury in MWF/Ztm rats.

An alternative explanation for the protective effect of the low

iron diet on renal injury in our model rests on the observation that iron is involved in the Haber-Weiss reaction promoting the formation of hydroxyl radicals [29]. Thus reduction of dietary iron could have decreased tissue iron, stored as ferritin, and consequent formation of oxygen free radicals that are source of potent permeability factors. Actually it has been reported that iron-dependent metabolites of hydrogen peroxide induce transient massive proteinuria and glomerular size-selective defect in the rat, and that free radical scavengers abolished hydrogen peroxide-induced proteinuria [30].

Experiments are necessary to clarify whether low iron diet is protective by virtue of changing glomerular microvascular shear stress, deriving from reduction in Hct, or whether the protection is mediated by a reduced generation of oxygen free radicals due to the low iron content of the diet. Independently from the mechanisms of its protective effect, the present data reinforce the concept previously put forward by Garcia and coworkers [5] that anemia, which frequently accompanies chronic renal failure, may be a protective factor against the renal disease progression. If this is the case the recent enthusiasm of correcting renal anemia in pre-dialysis patients with recombinant human erythropoietin should be tempered by the consideration that normalizing Hct in these patients may contribute to renal function deterioration.

In conclusion, reduction of Hct by a low iron diet has a protective effect on development of proteinuria and glomerulosclerosis in MWF/Ztm rats. Since determinants of glomerular ultrafiltration are not modified by diet-induced reduction in Hct, nor is glomerular hypertrophy prevented, we speculate that changes in blood viscosity and/or reduced generation of oxygen free radicals are the most likely factors involved in the observed effect of the diet. The present findings extend to another model the important previous observation that anemia is a renoprotective factor [5]. Such studies have obvious implications in the current management of pre-dialysis patients.

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